2011

Increased Tolerance to Abiotic Stresses in Tobacco Plants Expressing a Barley Cell Wall Peroxidase

Philippe Pringuet

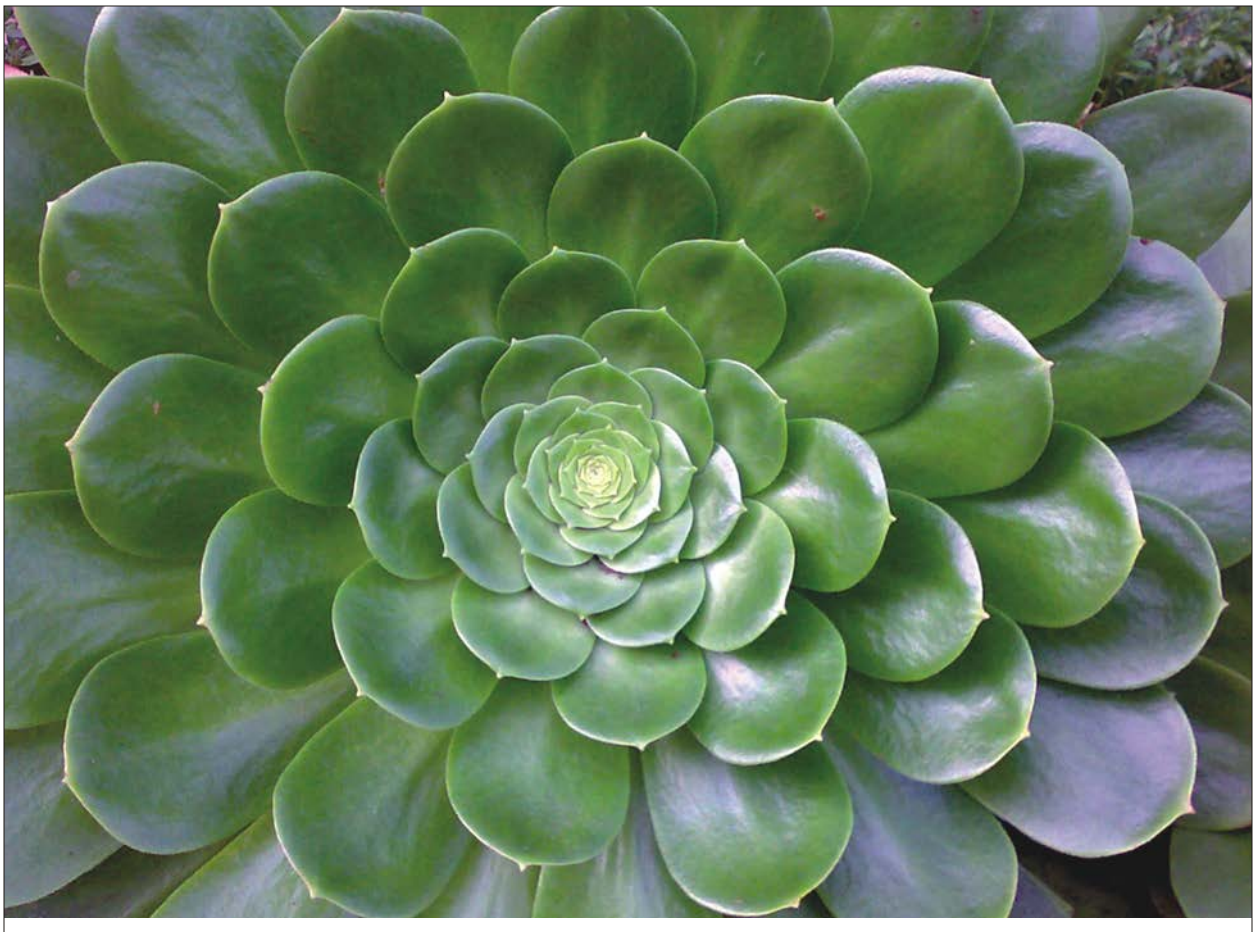
Norman McMillan

Greg Doyle

See next page for additional authors

Authors

Philippe Pringuet, Norman McMillan, Greg Doyle, Stephen Smith, and Sebastian Riedel



Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

Journal of Plant Sciences 6 (1): 1-13, 2011
ISSN 1816-4951 / DOI: 10.3923/jps.2011.1.13

Increased Tolerance to Abiotic Stresses in Tobacco Plants Expressing a Barley Cell Wall Peroxidase

[1]Deborah Vicuna, [2]Renée P. Malone and [3]Philip J. Dix

[1]Arkansas Biosciences Institute, Arkansas State University, P.O. Box 639, AR 72467, USA
[2]School of Food Science and Environmental Health, Dublin Institute of Technology, Dublin, Ireland
[3]Department of Biology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

Corresponding Author: Philip J. Dix, Department of Biology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland Tel: 353 1 7083836 Fax: 353 1 7083845

ABSTRACT

This study aimed to explore the prospects for enhancing abiotic stress tolerance through expression of a cell wall-targeted peroxidase in transgenic plants. Abiotic stresses result in the production of several Reactive Oxygen Species (ROS), including hydrogen peroxide (H_2O_2), in higher plants. H_2O_2 is highly diffusible and has a stress signalling role, but is also the source, through Fenton reactions, of highly destructive hydroxyl free radicals. Type III peroxidases, a family of heme-containing proteins which oxidise a range of substrates using H_2O_2 as oxidant, are capable of depleting H_2O_2 levels in several cellular compartments and specific peroxidases have been linked to stress defences. In the present study, we demonstrate expression of a pathogen-induced apoplastic barley peroxidase in transgenic tobacco plants and show that it confers improved tolerance to several abiotic stresses, including high and low temperatures, salinity, metal ion and osmotic stress.

Key words: Peroxidases, abiotic stress, tobacco, reactive oxygen species, salt stress, temperature stress

INTRODUCTION

Abiotic stresses, such as high and low temperatures, drought, salinity, heavy metals, high light and UV, exert a profound effect on the viability, growth, morphology and productivity of plants. Hence, these stresses have been inevitable targets for crop improvement through genetic engineering and the progress has been reviewed several times (Wang *et al*., 2003; Vinocur and Altman, 2005; Bhatnagar-Mathur *et al*., 2008). One strategy with potential to confer broad-range stress tolerance is to address the generation of reactive oxygen species (ROS, e.g., superoxide anions, O_2^- and hydrogen peroxide, H_2O_2 and the highly destructive, hydroxyl radical, $OH^\bullet$). These ROS have a high capacity for oxidation of lipids, nucleic acids and proteins, leading to extensive cell damage (Blokhina *et al*., 2003; Badawi *et al*., 2004). ROS are generated during normal metabolic processes, such as photosynthesis and cellular respiration, but their levels are generally controlled by a number of enzymes and anti-oxidants which have evolved for the purpose. The enzymes involved include Superoxide Dismutases (SODs), which convert O_2^- into H_2O_2 and a number of enzymes responsible for regulating H_2O_2 and minimising its conversion to $OH^\bullet$ via Fenton reactions. The latter enzymes include catalase and peroxidases, as well as components of cycles such as the water-water cycle, the ascorbate-glutathione cycle, or the glutathione peroxidase

cycle (Mittler, 2002) which depend on redox changes to the low molecular weight antioxidants, ascorbate and/or glutathione, for the reduction of H_2O_2. It is now well established that most abiotic stresses generate ROS to levels which may overwhelm these endogenous defense mechanisms and this is a major contributor to stress-mediated cellular damage (Mittler, 2002; Cheeseman, 2007).

Numerous efforts to engineer stress tolerance through manipulation of one or other of the ROS scavenging enzymes, have yielded frequently promising, but sometimes conflicting, results. For example, over-expression of SODs in the chloroplasts generally results in enhanced tolerance to one or more stresses (Bhatnagar-Mathur *et al*., 2008), but Tepperman and Dunsmuir (1994) found no effect of greatly increased SOD activity on oxidative stress tolerance and Bowler *et al*. (1991) observed that while large increases in chloroplast MnSOD levels increased oxidative stress tolerance, smaller increases were actually deleterious. These discrepancies doubtless relate to the complexity of the ROS-scavenging network and of the interactions between the different players, a feature of which is the signalling role of redox changes in the anti-oxidants and of the ROS themselves. The role of H_2O_2, is particularly challenging (Cheeseman, 2007). Clearly, as an oxidant in its own right, as well as the precursor for highly destructive $OH^{\bullet}$ radicals, it is a ROS to be controlled. However, it also has an important stress-signalling role (Neill *et al*., 2002), including retrograde signalling from the chloroplast (the principal site for ROS generation) to the nucleus, where it can regulate expression of stress-related nuclear genes (Nott *et al*., 2006). These functions are facilitated by the fact that it is relatively stable and can readily diffuse between cellular compartments (Neill *et al*., 2002). It is also a reactant in a wide range of biochemical reactions, including polyphenol metabolism and lignification (Lopez-Serrano *et al*., 2004) as well as auxin (IAA) metabolism (Gazaryan *et al*., 1996).

In view of the many facets of H_2O_2 action in plant cells, it is reasonable to suppose that a group of enzymes, the peroxidases, that utilise H_2O_2 to oxidase a range of substrates will also have multiple roles. Peroxidases are amongst the enzymes induced by the application of ROS-rich extracts, from drought-stressed leaves to rose plants (Gachomo and Simeon Kotchoni, 2008) and peroxidase activity has been correlated with salt tolerance in barley cultivars (Lilia *et al*., 2005) and in the halophyte *Soja sieb* (Li, 2009). There is a large number of peroxidase genes present in many plant species; for example at least 73 in *Arabidopsis*, of which transcripts of 58 could be detected (Welinder *et al*., 2002). This diversity of isoforms suggests individual isozymes have different roles in plant development and/or defence and are differentially regulated and targeted. Studies on over-expression of type III peroxidases in transgenic tobacco plants suggest that isozyme identity and cellular location can have a major impact on the phenotype. Jansen *et al*. (2004) explored the over-expression of a defence-related spruce peroxidase and a synthetic horseradish peroxidase in transgenic tobacco plants and found that the former had improved UV-tolerance, while the latter had reduced tolerance. Speculating on the reason for these contrasting results, the authors suggest that observed structural differences in the region of the heme binding pocket could effect substrate binding and intermediate stability which, combined with different cellular targeting (the spruce enzyme was believed to be apoplastic, while the horseradish enzyme was highly expressed in the cytosol, with smaller increases in the apoplast (Kis *et al*., 2004), could differentially effect phenolic and auxin metabolism. These metabolic perturbations could account for differences in plant morphology and lignification in the plants expressing the horseradish enzyme (Heggie *et al*., 2005).

These findings informed our choice of gene/enzyme for the current study aimed at increasing abiotic stress tolerance by over-expressing a peroxidase gene. There is a strong case for exploring an enzyme known to be highly induced in response to stress and with a well-characterised pattern

of expression and localisation. Two pathogen (powdery mildew)-induced peroxidases of barley, prx7 and prx8, exhibit distinct induction kinetics and subcellular localisation, vacuolar in the case of prx7, apoplastic in the case of prx8 (Kristensen *et al*., 1997). The authors demonstrate that prx8 over-expression reduces growth, but fails to increases pathogen resistance in transgenic tobacco, while a later transient expression study suggests prx7 expression can actually lead to reduced tolerance (Kristensen *et al*., 1999). Prx8 appears a good candidate for investigations into the prospects of improving abiotic stress tolerance through peroxidase over-expression, a view supported by a recent finding that this enzyme (but not prx7) is strongly up-regulated in response to abiotic (metal ion) stress in barley roots (Tamas *et al*., 2009). In this report we demonstrate that constitutive expression of apoplast-targeted barley peroxidase, prx8, confers improved tolerance to several abiotic stresses in transgenic tobacco.

MATERIALS AND METHODS

Location: The experimental work was performed in the Plant Cell Biology Laboratory, Biology Department, National University of Ireland, Maynooth, between 1st September 2001 to 31st March 2005.

Plant material: *Nicotiana tabacum* variety Xanthi was grown under 16/8 h light/dark photoperiod at 25°C. *In vitro* grown plants were maintained on MS medium (Murashige and Skoog, 1962) without added phytohormones and subcultured as nodal cuttings to fresh medium every 6 weeks. For seed production tobacco seedlings, rooted shoot cultures were planted in 8 inch pots filled with potting compost and grown in growth rooms at 22°C under the same day length conditions as described for the shoot cultures.

Seeds were surface sterilized in 10% domestic bleach (Domestos) for 10 min and germinated on half strength MS.

Vector construction: The barley peroxidase gene *prx8* was originally provided by Søren K. Rasmussen, Risø National Laboratory, Denmark. It was placed under control of the Cab promoter and terminator in a vector which included the kanamycin resistance gene, nptII, driven by the cauliflower mosaic virus 35S promoter (CaMV35S). The entire sequence was amplified by PCR, while adding a KpnI and a SacI restriction sites at either end of the gene. The sequence was excised using these sites and ligated into the final vector pBin19 (Fig. 1). DNA sequencing of individual clones confirmed the expected sequence and a clone was chosen for biolistic transformation.

Biolistic transformation: A PDS 1000/He Biolistic gene gun (BioRad) was used. For the bombardment, 1 μg of plasmid DNA was mixed with 50 μL of gold (0.6 μ, supplied by Biorad) stock

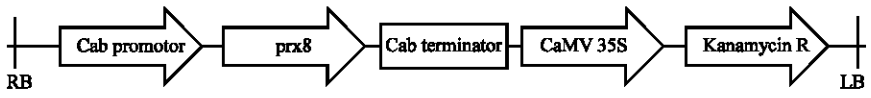


Fig. 1: Schematic representation of the T-DNA region of the vector used for transformation. The construct contained the prx8 gene driven by the Cab promoter and a selective marker gene for kanamycin resistance (KanR), driven by CaMV35S promoter

(40 mg mL^{-1}), 50 μL of $CaCl_2$ (2.5 M) and 20 μL Spermidine (0.1 M). The pellet was washed and resuspended in ethanol. Five microliter of the DNA mix was used per bombardment. The helium pressure was set to 1100 psi. The pressure used for the bombardment varied between 26-28 in Hg and the MS plates containing sterile (1 cm diameter) leaf discs the leaves were placed in the chamber at 6 cm below the sample holder. After bombardment, plates were incubated in darkness for 3 days and then transferred to regeneration medium (4.6 g L^{-1} MS salts + vitamins, 3% sucrose, 2 mg mL^{-1} naphthalene acetic acid, 0.25 mg mL^{-1} kinetin, mg mL^{-1} Thiamine HCl, pH 5.8 and 0.7% agar) containing 100 mg L^{-1} kanamycin and incubate under the usual conditions for shoot cultures. Kanamycin resistant shoots appeared after 4-6 weeks and were transferred to MS rooting medium containing 100 mg L^{-1} kanamycin sulphate.

DNA isolation, PCR and Southern analysis: Total genomic DNA was isolated from tobacco leaves according to Frey (1999). PCR analyses were carried out on 200-300 ng of genomic DNA using 5'ATG GCT TCT TCA TAC 3' and 5' TCA CGA GTT CAC CCT GGA GCA GCT 3' as primers. The initial step of 94°C for 4 min was followed by 25 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and one cycle of 5 min at 72°C. The PCR product was analyzed by electrophoresis on a 1% agarose gel in 1X TBE.

For Southern blots, 40 μg of DNA were digested with restriction enzyme HindIII overnight. This enzyme has a unique restriction site on the pCAS7/19-prx8 vector outside the prx8 gene and between the left and right border. This allows differentiation between transgenic plants resulting from separate events thanks to the size of the fragment hybridising to the probe as well as the distinction between independent integration sites within a transformant.

Non-radioactive Southern analysis was performed according to the method of McCabe *et al.* (1997). In order to obtain the probe, primers amplifying the complete cDNA sequence of the *prx*8 gene were used while incorporating DIG-labelled nucleotides. This probe was used to hybridise to the membrane containing the digested genomic DNA and to detect the presence of homologous sequence in the plant genome.

RNA analysis: RNA was isolated from leaf material by grinding with lysis buffer (0.1 M Tris HCl, pH 8.0, 50 mM EDTA, 50 mM NaCl, 2% SDS, 0.75% β-mercaptoethanol) and phenol. The lysate extracted once with phenol and twice with phenol:chloroform (1:1), finishing with one chloroform extraction. RNA was precipitated with 8M LiCl and washed with 0.1% DEPC-70% ethanol. RNA samples were treated with DNAse I and incubated for 25 min at 37°C. Samples were incubated with 25 mM EDTA for 15 min at 65°C. RT-PCR was performed with the isolated RNA. The Qiagen Omniscript™ Reverse Transcriptase kit for 1st strand cDNA synthesis was used.

Protein assays: Total soluble protein was extracted from plant material using extraction buffer (50 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, 1% Triton X-100, 1% PVP). The extract was filtered through two layers of Miracloth. Total protein was quantified according to the standard Biorad method, with BSA as the standard protein.

Peroxidase activity of all samples was determined using the substrate guaiacol. Fifty microgram of protein extract was mixed with a solution containing 0.3 mM guaiacol and 0.1 mM H_2O_2 in 100 mM Na_2HPO_4, pH 7.0. The increase in absorbance was followed for 3 min at 30 sec intervals at 436 nm. The rate of this reaction was used to express peroxidase activity. A Cary 1 E spectrophotometer was used for all enzyme assays.

Salt stress/metal stress treatments: Sterilized seeds were placed on Petri dishes containing MS medium plus 250 mM NaCl, 250 mM KCl, 470 mM mannitol for the salt stress or 5 or 10 mM $ZnCl_2$ and 5 or 10 mM $MnCl_2$ for the metal ions stress; or MS medium without additives. Three dishes with 40 seeds each were used per treatment. Germination was scored after 3 days, 1, 2 and 3 weeks. Seeds were considered to have undergone germination after the radicle and green cotyledons emerged.

Electrolyte leakage: Leaf discs (1 cm diameter) cut with a cork borer were obtained and exposed to 44°C for 6, 8 and 10 h or 4°C for 17, 24 or 48 h. The Electrolyte Leakage (EC) was measured immediately after the temperature stress ($EC_{initial}$) using the EC215 Conductivity meter (Hanna Instruments). The bathing solution containing the leaf discs was then boiled for 5 min and EC_{total} was measured. To obtain the Index of Injury (Id), the formula was used:

$$Id = 100\ (R_t\text{-}R_0)/(1\text{-}R_0)$$

where, $R_t = EC_{initial}/EC_{total}$ for stressed tissues and $R_0 = EC_{initial}/EC_{total}$ for nonstressed tissues.

Effect of heat stress on callus: Friable calli were obtained from leaf strips that were placed on callus induction medium (4.6 g L^{-1} MS salts medium, 3% sucrose, 2 mg mL^{-1} naphthalene acetic acid, 0.25 mg mL^{-1} kinetin, 1 mg mL^{-1} thiamine HCl, pH 5.8, 0.7% agar) and incubated at 25°C for 4 weeks. Cultures were exposed to 35, 40 and 44°C for 2, 4 and 6 h and then replaced in normal growth conditions for 3 weeks.

Heat stress of soil-grown plants: Two-week-old seedlings, germinated in vitro, were planted in soil and maintained under normal growth conditions for 2 weeks, then transferred to 40°C for one week. Plants were returned to normal temperature to recover for two further weeks.

RESULTS

Molecular analysis and gene expression in transgenic plants: All putative transformants were analyzed by PCR and DNA hybridization. Transcription of the transgene was confirmed by RT-PCR. PCR analyses performed with internal prx8 primers on DNA extracted from the transgenic lines showed the presence of the prx8 gene (data not shown). This was confirmed by Southern analysis (data not shown) that indicated multiple (2-5) inserts in the five independent lines examined. Stable integration and expression of the transgene was confirmed the five lines by reverse transcriptase-polymerase chain reaction (RT-PCR). Figure 2a shows the RT-PCR results for T0 plants. A band of 947 bp represents the cDNA synthesized from the RNA. This confirms the transcription of the transgene in the plants. No cDNA was present in the sample from wild type RNA.

Aside from the usual inter-plant variation in plants recovered from tissue culture, there were no differences in anatomy or growth rate between transgenic and wild type plants grown in soil after regeneration and propagation *in vitro* (T0 generation). Similarly, no differences were detected between the selfed progeny (T1 generation) of transgenic and wild type lines. The peroxidase activities of transgenic tobacco plants which tested positive for the insertion and transcription of the *prx*8 gene were determined in both T0 and T1 (selfed) plants. For T0 plants (data not shown) the five transgenic lines (GG03, GG05, GG06, GG07 and GG16), all showed higher foliar levels

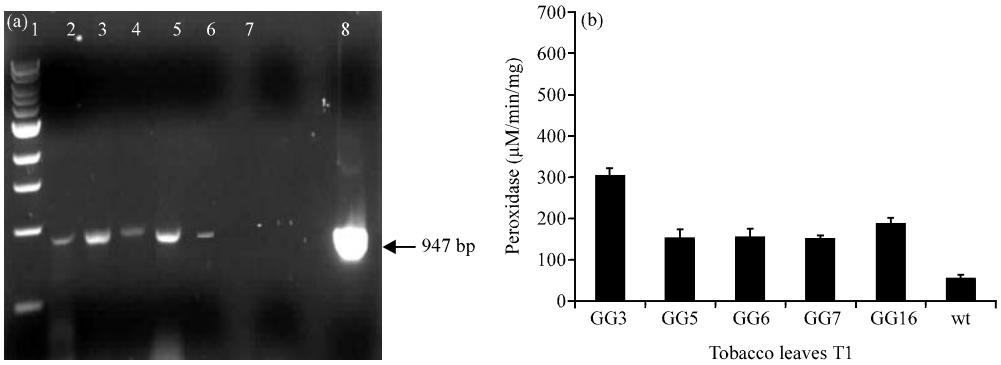


Fig. 2: Transcription and enzyme analysis of transgenic tobacco plants. RT-PCR products from T0 plants: Lane 1: 1 Kb ladder (Promega); lanes 2-6: positive transgenic tobacco lines GG03, GG05, GG06, GG07 and GG16; lane 7: wild type tobacco; lane 8: positive control (prx8 construct). Peroxidase activity was determined from leaf samples of 6 weeks old soil-grown plants. Samples are from T1 plants of transgenic lines: GG03, GG05, GG06, GG07 and GG16, as well as wild type (wt) tobacco samples. Results are presented in µM/min/mg. Data are the mean from three independent experiments

(from 2 fold up to a 4.5 fold increases) of peroxidase activity when compared to wild type plants, while levels in the roots were unchanged. Figure 2b shows the foliar peroxidase activities in T1 plants. In these samples, the levels of peroxidase were between 2.5 and 6 fold higher compared to the wild type. One line, GG03, consistently exhibited much higher levels of peroxidase activity than all the others and was chosen for all the following tests on stress tolerance.

Response to salinity and osmotic stress: Seeds from GGO3, as well as wild type tobacco seeds, were germinated on dishes of MS medium supplemented with 250 mM NaCl. In order to distinguish between specific ion (Na^+) and osmotic effects, seeds were also germinated on 250 mM KCl and 470 mM Mannitol. Figure 3 shows the results after three weeks. No differences were detected in germination rates or seedling growth when MS medium without added salts was used. However, seeds from the transgenic tobacco plants overexpressing the barley peroxidase gene exhibited higher germination rates on medium containing 250 mM NaCl than the wild-type plants. A similar pattern was obtained when KCl or Mannitol was added to the medium. About 40% of transgenic seeds growing on KCl and 25% growing on Mannitol germinated compared to only 2.5 and 5%, respectively for the wild-type.

Response to metal ion stress: Seeds from transformed homozygous tobacco plants (GGO3) expressing barley peroxidase were germinated on MS medium supplemented with $ZnCl_2$ and $MnCl_2$. Seeds from transformed and wild type tobacco germinated at the same frequency when MS medium without added salts was used. Seeds from the transgenic tobacco plants gave higher germination rates than the wild type plants on medium containing zinc and manganese at both 5 and 10 mM (Fig. 4a, b). More than 80% of transgenic seeds growing on 5 mM $ZnCl_2$ and $MnCl_2$ germinated compared to 20% for the wild type. Also, between 60 and 80% of the transgenic seeds germinated in the presence of 10 mM of the added salts compared to about 20% for the wild type seeds.

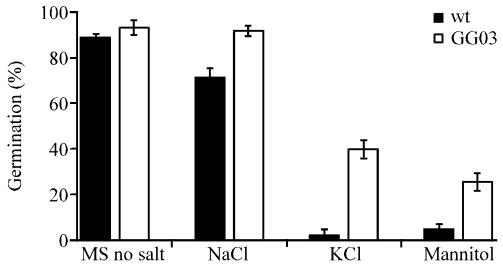


Fig. 3: Effect of salt and osmotic stress on tobacco seed germination. Germination of seeds from lineGG03 and wild type tobacco seeds, under salt and osmotic stress, was determined after 3 weeks. MS medium was supplemented with: 250 mM NaCl, 250 mM KCl or 470 mM mannitol. Germination was determined when radicle and green cotyledons were visible. Data are the means from three independent plates

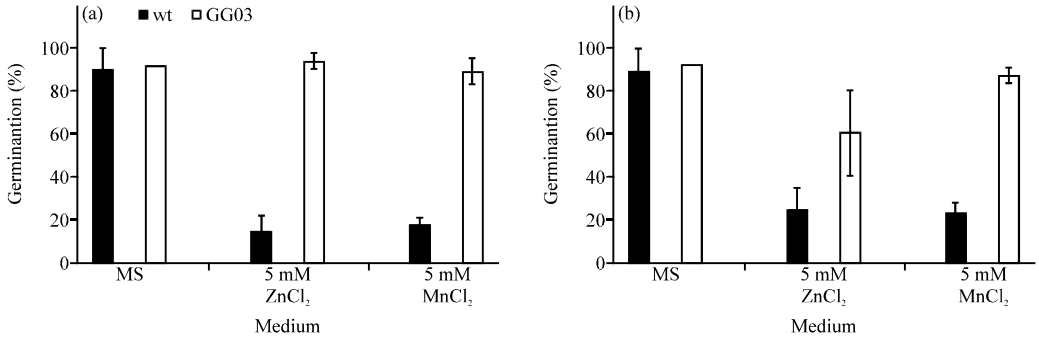


Fig. 4: Effect of metal ions on tobacco seed germination. Germination of seeds from lineGG03 and wild type tobacco seeds, under metal stress conditions, after 3 weeks. Seeds were germinated on MS medium supplemented with (a) 5 mM and (b) 10 mM, $ZnCl_2$ or $MnCl_2$. Germination was considered when radicle and cotyledons were visible. Data are the means from three independent plates

Response to high temperature stress: Electrolyte leakage was calculated using the conductivity of the bathing solution and referred to as the Index of Injury (Id). The Id was calculated using leaf discs incubated for 6, 8 and 10 h. Figure 5a shows the comparison of Ids between the wild type and the prx8 leaf discs and reveals smaller Ids for the prx8 plants after every incubation period. The low Ids indicate less membrane damage caused by the temperature stress. After 6 and 8 h, the wild type showed a 2 fold increase in the Ids when compared to the transgenic line. After 10 h, Ids of the transgenic line increased but the wild type's Ids remain higher, showing greater damage.

The consequences of peroxidase expression for heat tolerance were also explored in call us cultures and whole plants. Friable calli, initiated from leaves of T1 GGO3 plants, were exposed to 35, 40 and 44°C for 2, 4 and 6 h, transferred to normal growth conditions for 3 weeks and analyzed for their capacity to regenerate shoots. After exposure to 35 and 40°C, no differences were observed between the wild type and transgenic calli. However, after 6 h at 44°C, calli from the transgenic leaves showed an important difference when compared to the wild type. Wild type calli appeared necrotic, did not grow and failed to regenerate shoots (Fig. 5b). The transgenic calli appeared healthy, grew vigorously and regenerated several green shoots after 3 weeks.

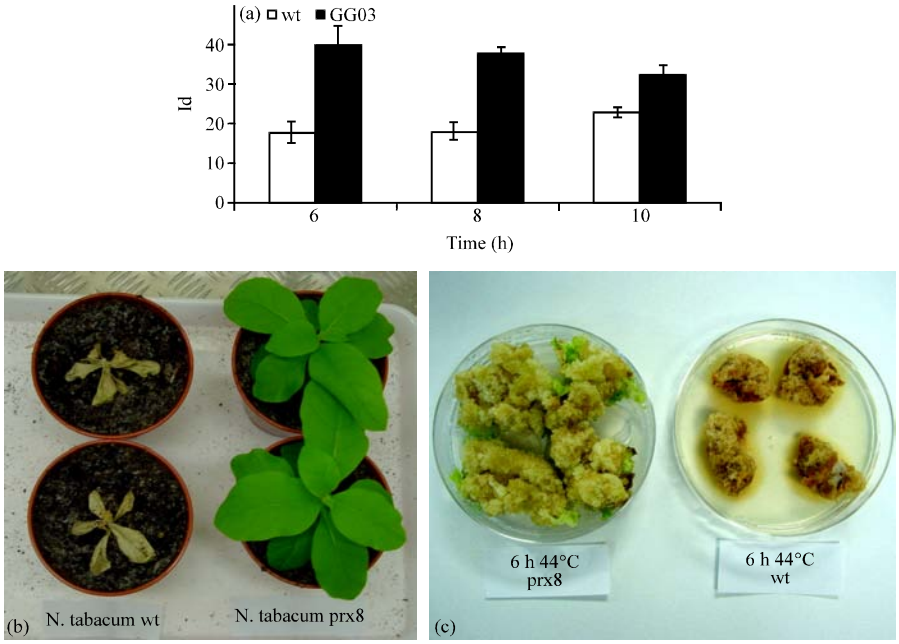


Fig. 5: Effect of heat stress on transgenic (line GG03) tobacco leaves, callus, and whole plants. (a) Index of Injury (Id) values obtained by conductivity measurements of leaf discs of T1 GG03 and wild type tobacco plants exposed to 44°C for 6, 8 and 10 h. Data are the means from three independent experiments. (b) Two-week old wild type (left) and T1 GG03 (right) tobacco plants were exposed to 40°C for 10 days and returned to normal growth conditions for a further 2 weeks, before being photographed. (c) Two-week old callus cultures from T1 GG03 (left) and wild type (right) plants were exposed to 44°C for 6 h and incubated at normal growth conditions for 3 weeks

Two-week old wild type tobacco plants and transgenic plants overexpressing the prx8 gene were stressed at 40°C for 10 days. They were then returned to normal growth conditions for 2 weeks before being compared for signs of damage by high temperature (Fig. 5c). Transgenic plants were able to recover from the heat stress and showed normal growth while, the wild type plants were killed.

Response to low temperature stress: Wild type tobacco plants and transgenic plants overexpressing the prx8 gene were tested for tolerance to low temperatures. Leaf discs were placed in deionised water and exposed to 4°C for 17, 24 and 48 h. Prx8 leaf discs showed insignificant damage when treated at this temperature after 17 and 24 h, while the wild type discs presented Index of injuries (Ids) of 9 and 8 respectively. After 48 h, the cold damage seemed to be present in both the wild type and the prx8 leave discs. Nonetheless, Ids for the wild type discs were twice as high as for the transgenic discs, indicating a lower tolerance to the temperature stress (Fig. 6).

DISCUSSION

In this report we describe the generation of transgenic tobacco plants containing a barley peroxidase gene, prx8, normally induced by fungal pathogens, but in our case placed under control of a leaf specific (chlorophyll a/b binding protein, Cab) promoter. The transgenic lines produced all

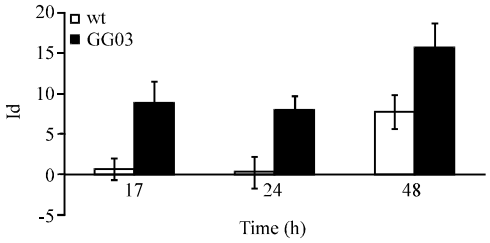


Fig. 6: Effect of cold stress on tobacco leaves, as determined by measurements of electrolyte leakage. Index of Injury (Id) values were obtained by conductivity measurements of leaf discs of T1 GG03 and wild type (wt) tobacco plants exposed to 4°C for 17, 24 and 48 h. Data are the means from three independent experiments

contained multiple copies of the transgene, transcription was demonstrated and a 2.5 to 6 fold increase in total soluble peroxidase activity was detected. The levels of elevated activity were similar to those achieved in several other studies in which different peroxidases have been over-expressed in *Nicotiana* species (Lagrimini *et al*., 1997; Kristensen *et al*., 1997; Kis *et al*., 2004). Kristensen *et al*. (1997), studying the expression of prx8, found it was accumulated to several folds the level of the native peroxidases, was secreted into the intercellular space and caused growth retardation but failed to improve resistance to powdery mildew infection. The current work was performed with the same construct (including the 21 amino acid apoplastic targeting peptide), obtained from those researchers. The plants obtained differ in two important respects, however. Kristensen *et al*. (1997) used *N. benthamiana*, not *N. tabacum*, as in the present study. This Australian species, is a widely used model for studies on plant-pathogen interactions (Goodin *et al*., 2008), but is quite distinct from *N. tabacum*. It is in the *Nicotiana* section *Suaveolentes*, restricted to Africa and Australasia, has a haploid chromosome number of 19 (compared to 24 in *N. tabacum*) and exhibits hybrid lethality in crosses with *N. tabacum* (Tezuka *et al*., 2010). It also favours arid environments unsuited to many *Nicotiana* species (Goodin *et al*., 2008) and therefore may exhibit more finely tuned molecular adaptations to stress than does tobacco. The second difference is that Kristensen *et al*. (1997) used a constitutive promoter (CaMV 35S) to drive expression of prx8, while we restricted expression to the photosynthetic tissue through the use of the Cab promoter, as indicated by the absence of elevated peroxidase activity in the roots. Kristensen *et al*. (1997) suggested the growth retardation observed in their plants is due to the ectopic expression of peroxidase promoting the cross-linking between cell wall polymers in cells which are still expanding. It is plausible that growing roots might be more susceptible than leaf cells to such modifications and that it is the use of a leaf specific promoter that prevented growth retardation in the current study.

For evaluation of the consequences of elevated peroxidase activity for abiotic stress tolerance, it was decided to focus on the transgenic line (GG03) that consistently gave the highest level of peroxidase activity (about 50% higher than any of the other lines). This line exhibited substantial improvements, compared to wild type tobacco, in tolerance to high and low temperatures, salinity, osmotic stress and toxic metal ions (Zn^{2+}, Mn^{2+}). Explanations for the improved tolerance could lie in improved ROS scavenging through the removal of H_2O_2 (Mittler, 2002), or in biochemical changes, e.g., in phenolic or auxin metabolism (Jansen *et al*., 2004) promoted by higher peroxidase activity. The diversity of abiotic stresses for which improved tolerance was obtained would appear to favour the former mechanism. All these stresses have been associated with ROS production and with the induction of peroxidases or other ROS scavenging enzymes. For example, heat stress

induces H_2O_2 and superoxide anions (O_2^-) in tobacco (Vacca *et al*., 2004) and peroxidase activity in species as diverse as mulberry (Chaitanya *et al*., 2002) and strawberry (Gulen and Eris, 2004). ROS have also been invoked in low temperature-induced cell damage (Wise and Naylor, 1987) and peroxidases are amongst the enzymes induced at low temperatures in tomato (Bruggemann *et al*., 1999) and cucumber (Lee and Lee, 2000). Peroxidase activity has also been shown to increase its levels in the presence of high salinity in *Vigna* seedlings (Pujari and Chanda, 2002) and Amaya *et al*. (1999) showed enhanced tolerance to salinity and osmotic stress in transgenic tobacco over-expressing a cell wall peroxidase. As with other abiotic stresses, it has been demonstrated that excess metal ions such as manganese (Gonzalez *et al*., 1998; Lidon and Teixeira, 2000) and zinc (Prasad *et al*., 1999) can induce the production of oxygen free radicals and consequently cause oxidative stress. Gonzalez *et al*. (1998) found an increased level of Superoxide Dismutase (SOD) while, Fang and Kao (2000) demonstrated the induction of peroxidase and showed that it was a direct effect of the metal ions and was not simply induced by the consequent elevation of H_2O_2 levels.

It appears that elevated type III peroxidases such as barley prx8, expressed in the apoplast, can confer improved tolerance to diverse abiotic stresses by minimising the accumulation of H_2O_2 and its subsequent breakdown to the toxic hydroxyl, $OH^\bullet$, radicle through Fenton reactions. This is despite the fact class III peroxidases are not normally considered at the front line of the ROS scavenging system, as are superoxide dismutase, catalase, ascorbate peroxidase and other enzymes in the water-water and ascorbate glutathione cycles (Mittler, 2002). While, the peroxidases clearly have important roles in plant development, their ability to scavenge stress-induced ROS, may only become important if the other defences fail. Mittler *et al*. (2004) describe how, in plants with knockout or antisense catalase or ascorbate peroxidase genes, the levels of class III peroxidases is elevated.

It is noteworthy that our improvements in stress tolerance are found in plants in which the peroxidase is targeted to the apoplast. Indeed earlier studies in which a synthetic horseradish peroxidase gene was preferentially expressed in the cytosol (Heggie *et al*., 2005) did not reveal any improvement in tolerance to some of the stresses (high and low temperatures) explored in the current study. If the main route to stress tolerance is ROS scavenging, it is not clear why apoplastic expression of a recombinant peroxidase should be more effective than cytosolic expression, where the enzyme should be closer to the intracellular sites of ROS generation.

Clearly, there is till a great deal to learn about the complex interplay between the various players in the defence of plants against the challenges posed by the environment and transgenic plants can be a valuable tool in dissecting the various interactions. It is clear from the present study however, that it is possible to achieve substantial improvements in abiotic stress tolerance through manipulation of a single component, in this case peroxidase, providing an appropriate isoenzyme is used and it is targeted to the correct tissue and cellular compartment. This augurs well as we confront new crop breeding challenges in the face of a changing environment.

ACKNOWLEDGMENTS

We would like to thank Dr. Soren Rassmussen at Risoe National Laboratory, Denmark for supplying the prx8 clone. Also, we would like to extend our appreciation to Dr. Elizabeth E. Hood, Distinguished Professor at Arkansas State University, for her encouragement and her invaluable assistance in the preparation of this manuscript. This study was funded by Dublin Institute of Technology R and D.

REFERENCES

Amaya, I., M.A. Botella, M. de la Calle, M.I. Medina and A. Herediaa *et al.*, 1999. Improved germination under osmotic stress of tobacco plants overexpressing a cell wall peroxidase. FEBS Lett., 457: 80-84.

Badawi, G.H., N. Kawano, Y. Yamauchi, E. Shimada, R. Sasaki, A. Kubo and K. Tanaka, 2004. Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco chloroplasts. Plant Sci., 166: 919-928.

Bhatnagar-Mathur, P., V. Vadez and K.K. Sharma, 2008. Transgenic approaches for abiotic stress tolerance in plants: Retrospect and prospects. Plant Cell Rep., 27: 411-424.

Blokhina, O., E. Virolainen and K.V. Fagerstedt, 2003. Antioxidants, oxidative damage and oxygen depravation stress: A review. Ann. Bot., 91: 179-194.

Bowler, C., L. Slooten, S. Vandenbranden, R. de Rycke and J. Botterman *et al.*, 1991. Mangenes superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J., 10: 1723-1732.

Bruggemann, W., V. Beyelb, M. Brodka, H. Poth, M. Weiland and J. Stockhaus, 1999. Antioxidants and antioxidative enzymes in wild-type and transgenic *Lycorpersicon* genotypes of different chilling tolerance. Plant Sci., 140: 145-154.

Chaitanya, K.V., D. Sundar, S. Masilamani and A. Reddy, 2002. Variation in heat stress-induced antioxidant enzyme activities among three mulberry cultivars. Plant Growth Regul., 36: 175-180.

Cheeseman, J.M., 2007. Hydrogen peroxide and plant stress: A challenging relationship. Plant Stress, 1: 4-15.

Fang W.C. and C.H. Kao, 2000. Enhanced peroxidase activity in rice leaves in response to excess iron copper and zinc. Plant Sci., 158: 71-76.

Frey, J.E., 1999. Genetic flexibility of plant chloroplasts. Nature, 398: 115-116.

Gachomo, E.W. and O. Simeon Kotchoni, 2008. Extract from drought-stress leaves enhances disease resistance through induction of pathogenesis related proteins and accumulation of reactive molecules. Biotechnology, 7: 273-279.

Gazaryan, I.G., L.M. Lagrimini, G.A. Ashby and R.N. Thorneley, 1996. Mechanism of indole-3-acetic acid oxidation by plant peroxidases: Anaerobic stopped-flow spectrophotometric studies on horseradish and tobacco peroxidases. Biochem. J., 313: 841-847.

Gonzalez, A., K.L. Steffen and J.P. Lynch, 1998. Light and excess manganese. Implications for oxidative stress in common bean. Plant Physiol., 118: 493-504.

Goodin, M.M., D. Zaitlin, R.A. Naidu and S.A. Lommel, 2008. *Nicotiana benthamiana*: Its history and future as a model for plant-pathogen interactions. Mol. Plant Microbe. Interact., 21: 1015-1026.

Gulen, H. and A. Eris, 2004. Effect of heat stress on peroxidase activity and total protein content in Strawberry plants. Plant Sci., 166: 739-744.

Heggie, L., M.A. Jansen, E.M. Burbridge, T.A. Kavanagh, R.N. Thorneley and P.J. Dix, 2005. Transgenic tobacco (*Nicotiana tabacum* L. cv. Samsun-NN) plants over-expressing a synthetic HRP-C gene are altered in growth, development and susceptibility to abiotic stress. Plant Physiol. Biochem., 43: 1067-1073.

Jansen, M.A.K., M. Elfstrand, L. Heggie, F. Sitbon, P.J. Dix and R.N.F. Thorneley, 2004. Over-expression of phenol-oxidising peroxidases alters the UV-susceptibility of transgenic *Nicotiana tabacum*. New Phytol., 163: 585-594.

Kis, M., E. Burbridge, I.W. Brock, L. Heggie, P.J. Dix and T.A. Kavanagh, 2004. An N-terminal peptide extension results in efficient expression, but not secretion, of a synthetic horseradish peroxidase gene in transgenic tobacco. Ann. Bot., 93: 303-310.

Kristensen, B.K., J. Brandt, K. Bojsen, H. Thordal-Christensen and K.B. Kerby *et al.*, 1997. Expression of a defence-related intercellular barley peroxidase in transgenic tobacco. Plant Sci., 122: 173-182.

Kristensen, B.K., H. Bloch and S.K. Rasmussen, 1999. Barley coleoptile peroxidases. Purification, molecular cloning and induction by pathogens. Plant Physiol., 120: 501-512.

Lagrimini, L.M., V. Gingas, F. Finger, S. Rothstein and T.T.Y. Liu, 1997. Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. Plant Physiol., 114: 1187-1196.

Lee, D.H. and C.B. Lee, 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: In gel enzyme activity assays. Plant Sci., 159: 75-85.

Li, Y., 2009. Effects of NaCl stress on antioxidative enzymes of glycine soja sieb. Pak. J. Biol. Sci., 12: 510-513.

Lidon, F.C. and M.G. Teixeira, 2000. Oxyradicals production and control in the chloroplast of Mn-treated rice. Plant Sci., 152: 7-15.

Lilia, E., S. Rezgui, H. Slim Amara, A. Daaloul, M. El Hassni and I. El Hadrami, 2005. Effect of salt treatment on the expression of phenolics and peroxidase activity assessed in two barley cultivars acsad 1230 and Arig 8. J. Agron., 4: 196-202.

Lopez-Serrano, M., M.D. Fernandez, F. Pomar, M.A. Pedreno and A.R. Barcelo, 2004. *Zinnia elegans* uses the same peroxidase isoenzyme complement for cell wall lignification in both single-cell tracheary elements and xylem vessels. J. Exp. Bot., 55: 423-431.

McCabe, M.S., J.B. Power, A.M. de Laat and M.R. Davey, 1997. Detection of single-copy genes in DNA from transgenic plants by nonradioactive Southern blot analysis. Mol. Biotechnol., 7: 79-84.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci., 7: 405-410.

Mittler, R., S. Vanderauwera, M. Gollery and F. van Breusegem, 2004. Reactive oxygen gene network of plants. Trends Plant Sci., 9: 490-498.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Neill, S., R. Desikan and J. Hancock, 2002. Hydrogen peroxide signalling. Curr. Opin. Plant Biol., 5: 388-395.

Nott, A., H.S. Jung, S. Koussevitzky and J. Chory, 2006. Plastid-to-nucleus retrograde signalling. Annu. Rev. Plant Biol., 57: 739-759.

Prasad, K.V.S.K., P.P. Saradhi and P. Sharmila, 1999. Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*. Environ. Exp. Bot., 42: 1-10.

Pujari, D.S. and S.V. Chanda, 2002. Effect of salinity stress on growth, peroxidase and IAA oxidase activities in vigna seedlings. Acta Physiol. Plant, 24: 435-439.

Tamas, L., K. Durcekova, J. Huttova and I. MistrIk, 2009. Expression of defence-related peroxidases prx7 and prx8 during abiotic stresses in barley roots. Acta Physiol. Plant, 31: 139-144.

Tepperman, J. and P. Dunsmuir, 1994. Transformed plants with elevated levels of chloroplastic SOD are not more resistant to superoxide toxicity. Plant Mol. Biol., 14: 501-511.

Tezuka, T., T. Kuboyama, T. Matsuda and W. Marubashi, 2010. Seven of eight species in *Nicotiana* section *Suaveolentes* have common factors leading to hybrid lethality in crosses with *Nicotiana tabacum*. Ann. Bot., 106: 267-276.

Vacca, R.A., M.C. de Pinto, D. Valenti, S. Passarella, L. Marra L.and L. de Gara, 2004. Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase and impairment of mitochondrial metabolism are early events in heat shock induced programmed cell death in tobacco bright-yellow 2 cells. Plant Physiol., 134: 1100-1112.

Vinocur, B. and A. Altman, 2005. Recent advances in engineering plant tolerance to abiotic stress: Achievements and limitations. Curr. Opin. Biotechnol., 16: 123-132.

Wang, W., B. Vinocur and A. Altman, 2003. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta, 218: 1-14.

Welinder, K.G., A.F. Justesen, I.V. Kjaersgard, R.B. Jensen, S.K. Rasmussen, H.M. Jespersen and L. Duroux, 2002. Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana*. Eur. J. Biochem., 269: 6063-6081.

Wise, R.R. and A.W. Naylor, 1987. Chilling-enhanced photooxidation: Evidence for the role of singlet oxygen and superoxide in the breakdowm of pigments and endogenous antioxidants. Plant Physiol., 83: 278-282.